Immunity, Volume *25*

Supplemental Data

Regulatory T Cells Reversibly Suppress

Cytotoxic T Cell Function

Independent of Effector Differentiation

Thorsten R. Mempel, Mikael J. Pittet, Khashayarsha Khazaie, Wolfgang Weninger, Ralph Weissleder, Harald von Boehmer, and Ulrich H. von Andrian

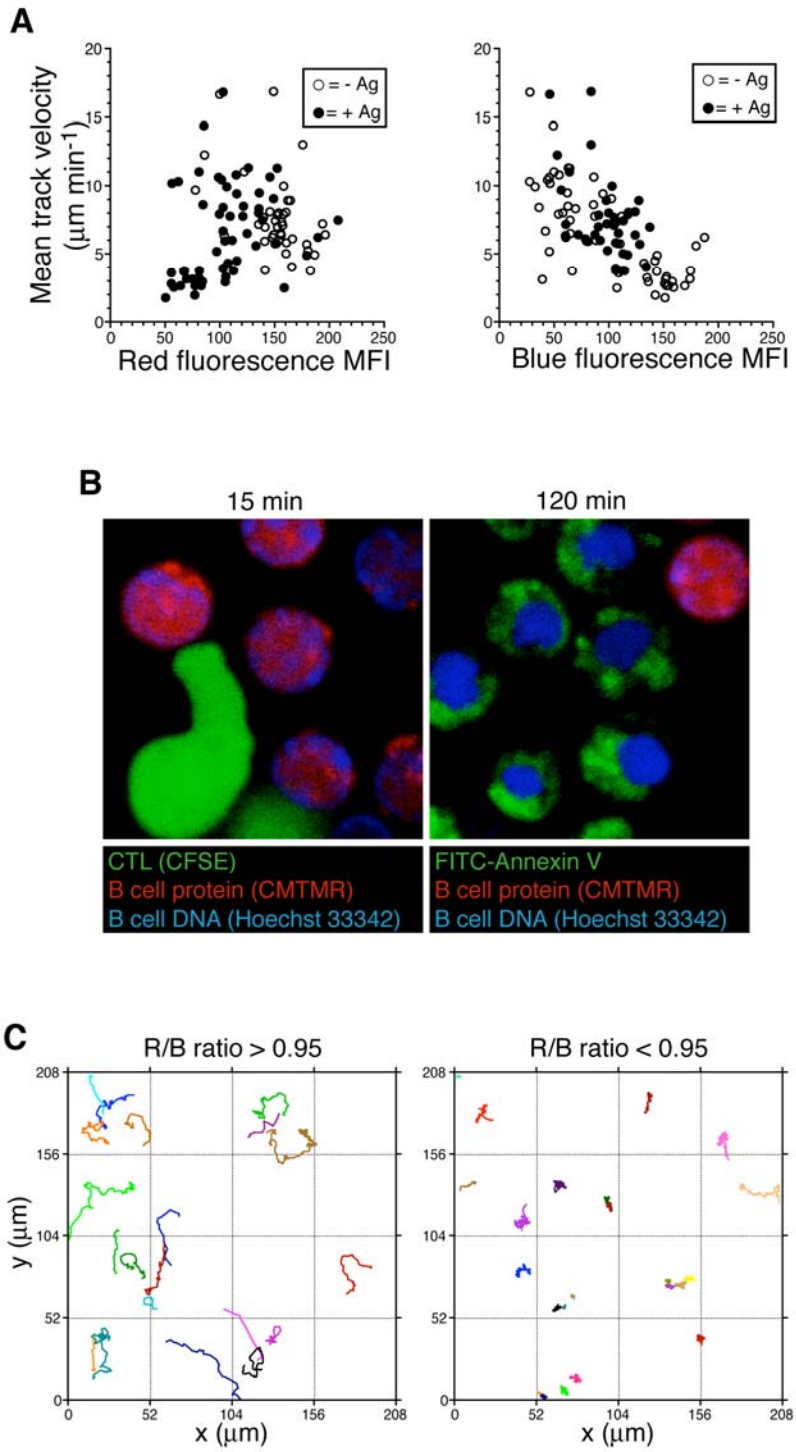

Figure S1. Change in Fluorescence Properties and Loss of Motility Identify Lysed B Cells

(A) Red and blue mean fluorescence intensity (MFI) of single Ag-free (empty symbols) and Ag-pulsed (filled symbols) B cells were determined from MP-IVM recordings in tumor-draining LNs 2 to 5 hr after adoptive transfer. Data are plotted against the cells' mean track velocity.

(B) In vitro-generated HA-specific CTLs and $HA_{512-520}$ peptide-pulsed B cells were labeled with CFSE and CMTMR/Hoechst33342, respectively, and coincubated in vitro at 37°C in medium containing soluble FITC-Annexin

V. Representative multiphoton micrographs were taken after 15 min (left) and 120 min (right) of coculture. The left panel depicts polarization of a CTL (green) toward a target B cell (red/blue). After 120 min, apoptotic or dead target cells have undergone a loss of red fluorescence concomitant with increased and more homogenous blue fluorescence as well as Annexin V reactivity (green). The decrease in red fluorescence is likely a result of loss of soluble cellular protein and the increase in blue fluorescence from alterations in spectral properties or unquenching of fluorescent Hoechst33342 molecules.

(C) Representative tracks in xy view from a 1 hr recording (taken at 4 hr after B cell injection) of Ag-pulsed B cells with R/B ratio fluorescence either >0.95 (left) or <0.95 (right). Minor displacements of B cells with an R/B ratio <0.95 likely reflect passive movement through surrounding motile cells. Most B cells with an R/B ratio of >0.95 exhibited a high motility and migrated out of the recorded region before the end of the recording.

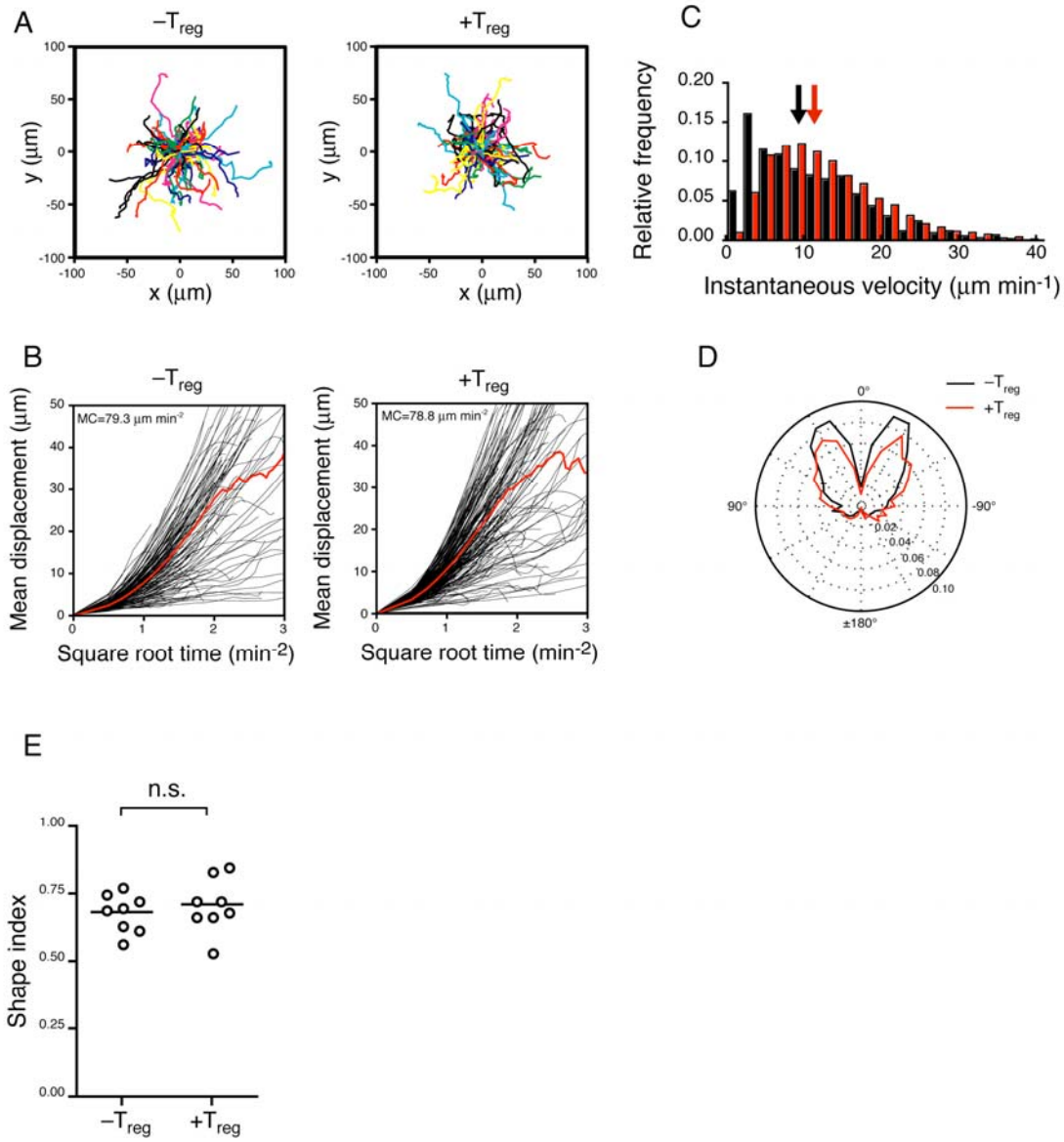

Figure S2. Presence of T_{reg} Cells Does Not Alter the Migratory Behavior of CTLs in LNs

(A) Normalized migratory tracks of nonregulated (left) and regulated (right) CTLs before injection of B cells.

(B) Single cell (black lines) and population (red lines) mean displacement (MD) plots for the same data sets as in (A). MC, motility coefficient, derived from the linear segment of the population MD plot.

(C) Relative frequency distributions of instantaneous migratory velocities for the same data set as in (A). The median velocity for regulated CTLs (11.34 µm min^{-1}; red bars and arrow) was slightly higher than for nonregulated CTLs (9.11 µm min^{-1}; black bars and arrow).

(D) Turning behavior of migrating nonregulated (black) and regulated (red) CTLs for the same data set as in (A) and depicted as radial relative frequency plots.

(E) Average 2D shape indices (20 measurements per cell) were calculated as $S = 4\pi$ area/$perimeter^2$ for 8 randomly selected CTLs per group during their motile interaction with Ag-pulsed B cells in the absence and presence of T_{reg} cells ($p = 0.53$).

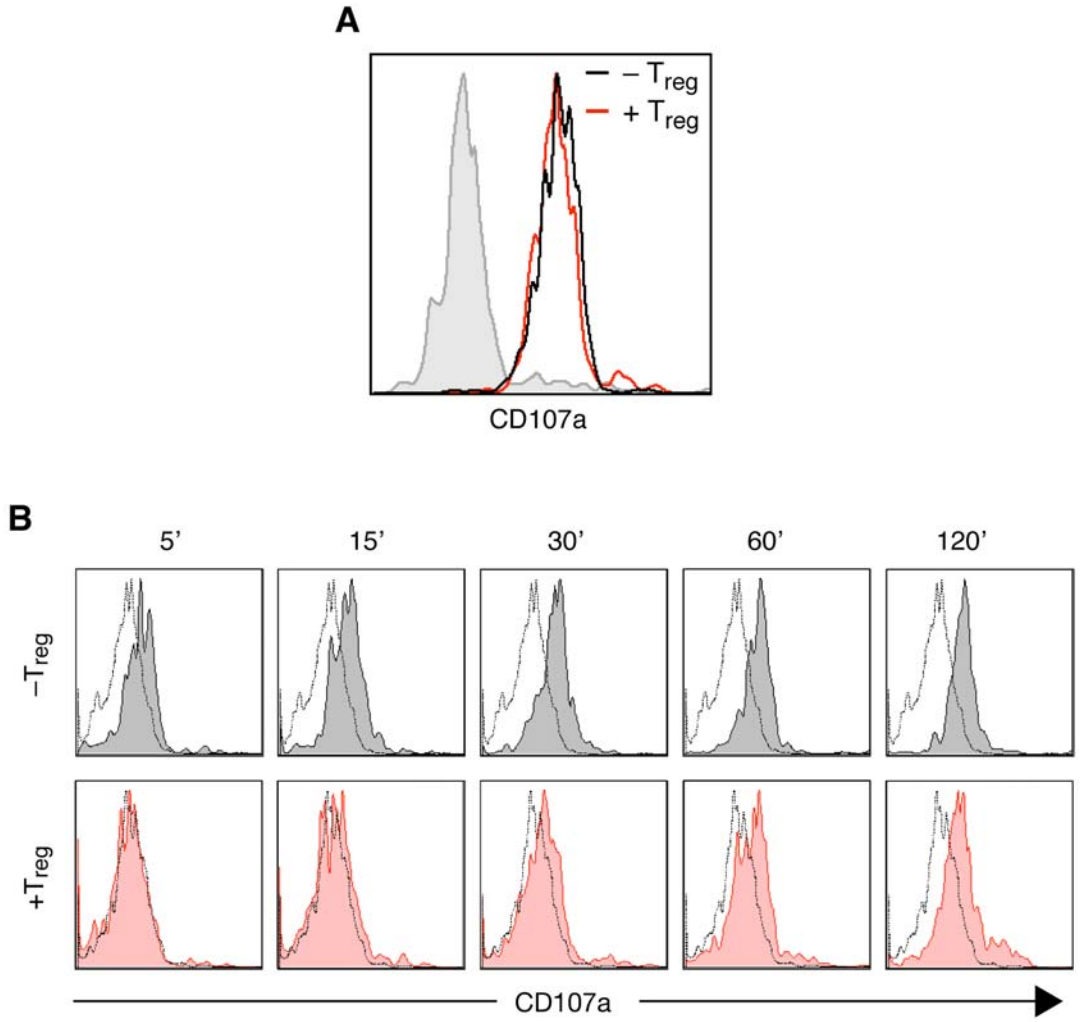

Figure S3. CTL Granule Exocytosis in Response to Ag Challenge Is Delayed in the Presence of T_{reg} Cells

(A) Permeabilized LN-derived CTLs obtained on day 6 of the immune response were stained intracellularly for the lysosomal membrane marker CD107a and analyzed by flow cytometry. Regulated (red line) and nonregulated (black line) HA-specific CTLs contained the same amount of CD107a. Isotype-matched control mAb staining is shown in gray.

(B) The kinetics of CTL degranulation were determined by measuring cell surface mobilization of CD107a in nonregulated (top row) and regulated CTLs (bottom row) at different time points after addition of $HA_{512-520}$ peptide and CD107a mAb. Granule exocytosis was considerably slowed in regulated CTLs, in particular during the first 30 min after Ag challenge. Dashed histograms show background levels of CD107a staining before addition of Ag. Similar background staining was observed after 120 min of culture in the absence of HA peptide (not shown).

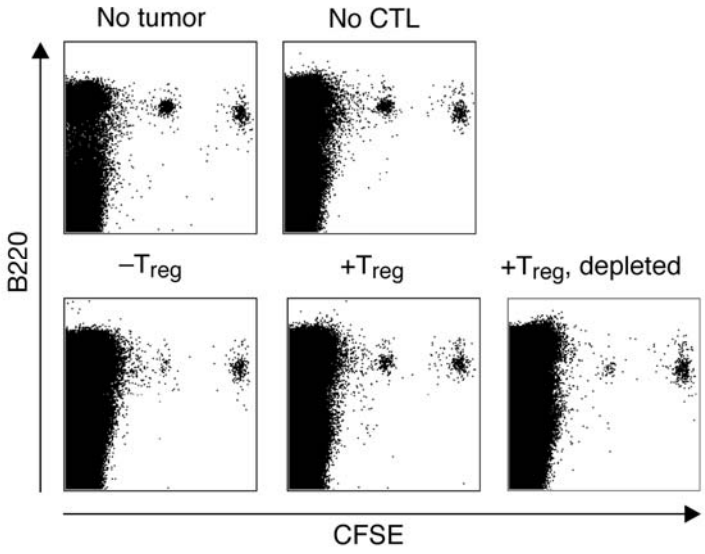

Figure S4. Suppression of CTL Function by T_{reg} Cells Is Reversible

Thy1.1^{+} T_{reg} cells were depleted from Thy1.2^{+} mice as described in Figure 6A. Killing activity in tumor-draining LNs was measured on day 8 by intravenously injecting a 1:1 mixture of syngeneic B cells previously pulsed or not with $HA_{512-520}$ and labeled with two different concentrations of CFSE. 6 hr later, the relative decrease in number of Ag-pulsed B cells (CFSE^{low}) compared to Ag-free control B cells (CFSE^{high}) was measured by flow cytometry. Mice that had received neither tumors nor T cells were used to correct for differential homing of pulsed and unpulsed B cells in LNs. This representative example demonstrates that regulated CTLs acquire potent cytotoxic activity upon selective depletion of T_{reg} cells ("+T_{reg}, depleted"). Tumor-free mice ("no tumor") as well as tumor-bearing mice in which T_{reg} cells were not depleted ("+T_{reg}") and tumor-bearing mice that received CTLs only ("–T_{reg}") or no T cells at all ("no CTL") are shown as controls.

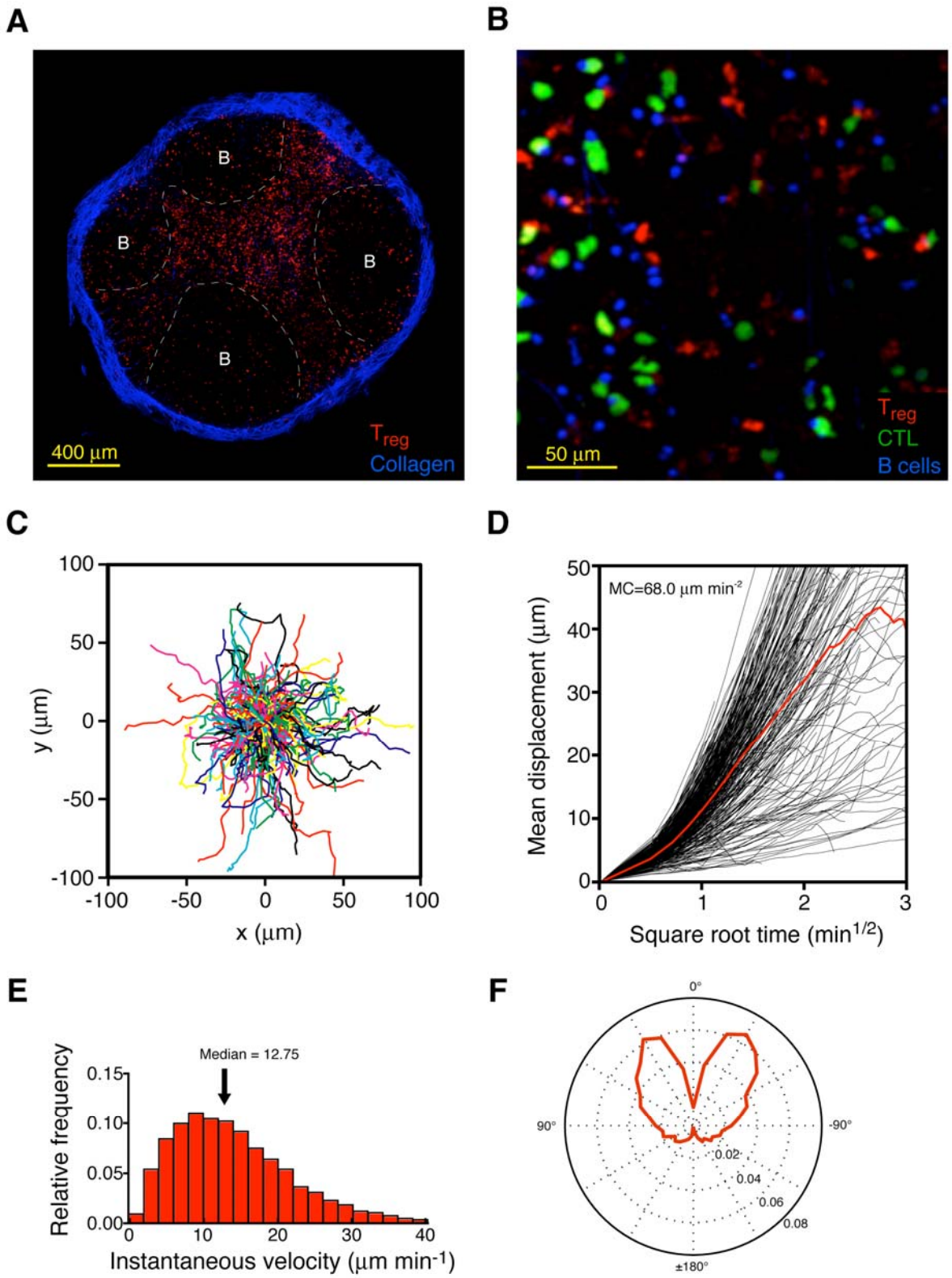

Figure S5. Location and Motility of T_{reg} Cells in LN

(A) Composite MP-IVM micrograph of a tumor-draining popliteal LN. Activated T_{reg} cells (red) reside primarily in the T cell area, but are also capable of entering B cell follicles (delineated by broken lines). Second harmonic signals from collagen fibers are shown in blue.

(B) Intravital micrograph showing CTLs (green) and T_{reg} cells (red) colocalizing in the same area as Ag-pulsed B cells (blue).

(C) Normalized migratory tracks of Treg cells.

(D) Single-cell (black lines) and population (red line) mean displacement plots for the same data sets as in (C). MC, motility coefficient.

(E) Frequency distributions of instantaneous migratory velocities for the same data set as in (C).

(F) Turning behavior of migrating T_{reg} cells for the same data set as in (C) and depicted as radial relative frequency plots. These data characterize T_{reg} cells as a highly motile leukocyte population with similar migratory characteristics as activated $CD8^+$ T cells.

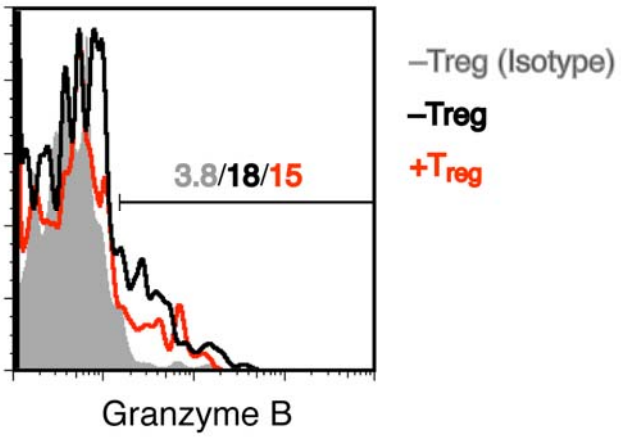

Figure S6. Intracellular Granzyme B Content of Regulated and Unregulated CTLs

Flow cytometry histograms of regulated (red line) and nonregulated (black line) CTLs obtained from tumor-draining LNs on day 7 post tumor challenge stained for intracellular 1 granzyme B with and anti-human granzyme B mAb cross-reactive with mouse granzyme B (Clone GB11). Gray, solid histogram shows background staining of unregulated CTLs stained with isotype-matched control mAb.